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From the Institute of Virology of the Veterinary Highschool, Hannover. Director: Prof. Dr. B. Liess.

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INVESTIGATIONS FOR THE DETECTION OF NEUTRALIZING ANTIBODIES AGAINST THE VIRUS OF BOVINE VIRUS DIARRHEA-MUCOSAL DISEASE (BVD-MD) AS WELL AS PRECIPITATING ANTIBODIES AGAINST A PURIFIED VIRUS ANTIGEN OF EUROPEAN HOG CHOLERA (EHC) IN THE BLOOD SERUM OF PIGS USING THE MICROPRECIPITATION TECHNIQUE

By

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CONTENTS

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Abbreviations

BVD-MD	Bovine virus diarrhea-mucosal disease.
EHC	European hog cholera
FCK	Foetal calf kidney
FCS	Foetal calve serum
g.	Acceleration due to gravity
CID_{50}	50% culture infective dose
NIF	Neutralization and immunofluorescence test
N-test	Virus neutralization test
n.e	not examined
PFU	Plaque-forming unit
PBS	Phosphate buffer solution, after Dulbecco and Vogt (1954
NS_{50}	50% neutralizing serum dose
rpm	revolution per minute
CPE	Cytopathic effect

1. Introduction

The identity lines detected in the agar-gel precipitation test (AGP) between an antiserum against the virus of the European hog cholera (EHC) and antigens prepared from the organ extracts of affected cattle or pigs referred, for the first time, to the existence of an antigenic relationship between the virus of the bovine virus diarrhea-mucosal disease (BVD-MD) and that of the EHC (Darbyshire, 1960, 1962). This phenomenon of the common soluble virus-specific antigens was confirmed by Malmquist and Gutekunst (1963). By means of immunoelectrophoresis Matthaeus and Van Aert (1971) showed that 3 of the 5 precipitation lines obtained with antigen extracts from porcine pancreas were related to the BVD-MD antigens. This antigenic relationship of both types of viruses was also detected by the complement-fixation test (CFT) (Gutekunst and Malmquist, 1963) immunofluorescence (Mengeling et al., 1963) as well as the neutralization test (Mengeling et al., 1963).

These correlations led also to cross-immunization trials in order to protect pigs from infection with the EHC virus by means of administration of BVD-MD virus (Sheffy et al., 1962; Beckenhauer et al., 1961). Nevertheless, sera of pigs which have been infected with the BVD-MD virus showed no neutralizing antibodies against the EHC-virus, but only against the BVD-MD virus. Following administration of the EHC virus to these pigs, the development of EHC virus-neutralizing antibodies was accompanied by a rise of the BVD-MD-antibody titer (Sheffy et al., 1962). Later on, Snowdon and French (1968) found differences among different BVD-MD virus strains concerning their ability to induce the production of EHC virus-neutralizing antibodies in pigs. These authors mentioned that the proportion of the BVD-MD and EHC-antibody titers can be dependent upon the number of cell culture passages, and hence, they confirmed the results of Sheffy et al., (1962) indirectly, according to which the effectivity of the BVD-MD virus as a protecting agent against EHC will be decreased with an increasing number of cell culture passages (a translation made by the author). Snowdon and French (1968) reported—unfortunately without any details— that 60 out of 77 pigs which have been subjected to a challenge test with the EHC virus and tolerated it, revealed the presence of BVD-MD— or EHC-antibodies either separately or collectively; 85% of the 60 pigs had BVD-MD-neutralizing antibodies. Up till now,

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little is known about the significance of this limited finding for the epizootiology of the EHC infection and, particularly, the origin of the BVD-MD antibodies in the swine populations, although Phillip and Darbyshire (1972) infected pigs with the BVD-MD virus without detecting any clinical manifestations, where they isolated the virus from different organs on the 20th day postinoculation.

This investigation was dedicated to the examination of a large number of swine sera from piglet- rearing forms, in which anamnestic neutralizing antibodies have been previously detected against EHC virus (NIF-positive) as well as sera from NIF-negative herds for the presence, frequency and titer of BVD-MD neutralizing antibodies. Moreover, I had the opportunity of testing the same sera for the presence of precipitating antibodies against EHC antigen aiming at a comparative evaluation of the diagnostic value of both methods. The Lower-Saxony Minister for Nutrition, Agriculture and Forestry requested the Director of the Institute of Virology of the Veterinary Highschool at Hannover, Prof. Dr. Liess, to give his opinion on the idea that the precipitation test represents the method of choice for the serological screening of EHC antibodies.

2. Author's Examination

2.1. Material and Methods

2.1.1. Serum samples

A total of 420 swine sera from 39 piglet-rearing farms, which have been used before in previous investigations performed in 3 regions of a district in Lower-Saxony (Liess et al., in preparation), was available for these examinations. Outbreaks of EHC have been detected 2 years ago in all these 3 regions, before the collection of blood samples. In the screening test, serum samples have only been collected from those animals fit for breeding purposes. At the time of collecting the blood samples, and according to the informations given by the responsible veterinary officer, the herds were free of any clinical manifestation of the EHC.

The number of samples collected from each herd varied according to the size of the herd (see Table).

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Author's Experiments

Material and Methods

[illegible] samples

[illegible] total of 429 swine [illegible] eight-[illegible] been used before in previous [illegible] [illegible] of a district in Lower-Saxony [illegible] [illegible], was available for these [illegible] have been detected [illegible] years ago in all [illegible] collection of blood samples. [illegible] samples have only been collected [illegible] [illegible]. At the time of collecting the blood samples, [illegible] to the informations given by the [illegible] [illegible] officer, the herds were free of any clinical manifestation [illegible]

[illegible] of samples collected from each herd [illegible] the size of the herd (see Table[illegible]).

Size of the herd	Number of collected blood samples
1- 5 breeding animals	from all animals
5-20 breeding animals	at least from 5 animals
over 20 breeding animals	at least from 25% of the animals

As a matter of fact, the average number of the random samples collected per herd was about 39%.

In those herds which proved to contain NIF-positive pigs, according to the random examinations, blood samples were collected from all animals fit for breeding for further examinations. This explains the fact that a larger number of serum samples, from the NIF-positive herds, was available for examination than that obtained from the NIF-negative animals (see appendix 6.1).

The origin of blood samples collected from each of those areas, in which clinically manifested EHC outbreaks have been detected during the last years, shows that the groups of blood samples were not accidentally selected.

Besides the blood sera from those 39 herds, which have been selected by preliminary examinations, another 37 sera from a further herd which proved to be positive on an official follow-up examination were also included in our examination.

All blood samples were centrifuged in a laboratory centrifuge[1] for 10 minutes at 1000 rpm, and the obtained sera were, then, kept at -20°C till examination. These sera were examined in the dilution 1:5 by the NIF test for the presence of EHC-neutralizing antibodies,if any neutralizing activity was detected, the positive sera were titrated. The results of the examinations for the presence of EHC-neutralizing antibodies have been already available since the beginning of the examinations, conducted for comparative purposes in our investigation. Accordingly, a total of 457 swine sera from 40 herds were available (39 herds with 420 sera from a screening test, as well as 37 sera from a herd on which follow-up

1) MSE, Mistral

examinations have been carried out). Table 1 presents the classification of these groups of sera according to the number of the farms with NIF-positive animals (NIF-positive herds) and the number of animals in these farms, as well as according to whether farms are with or without cattle-housing on the same premises (this is of significance for the possible transmission of the BVD-MD virus to the pigs).

<u>Table 1</u>

Number of swine sera available for examination for the presence of BVD-MD neutralizing antibodies, classified according to the origin of the swine-raising farms, with or without cattle-housing on the same premises, as well as according to the suspected presence of EHC (NIF-positive sera from a preliminary examination), or unsuspicious as indicated from preliminary and follow-up examination.

1	2		3		4		5
Art des Herkunftsbetriebes[1] a	b Gesamtzahl[3] geprüfter		Gesamtzahl der Seren aus NIF-negativen Beständen e		Gesamtzahl der Seren aus NIF-positiven Beständen[2] f		% Seren aus NIF-positiven Beständen g
	Seren c	Bestände d	Seren	Bestände	Seren[4]	Bestände	
A	276	24	56	10	220	14	79,7
B	166	16	55	10	111	6	66,9
A + B	442	40	111	20	331	20	74,9
Seren pro Betrieb h	11,1		5,5		16,6		

1) Farm with (A) or without (B) cattle-housing

2) Neutralizing activity against the EHC virus in the serum dilution 1:5, detected by the NIF test

3) Inclusive NIF-negative sera from NIF-positive herds

4) Nonincidental random samples (see 2.1.1.).

<u>Key:</u>
a) Farm of origin; b) Total number examined; c) Sera; d) Herds; e) Total number of sera from NIF-negative herds; f) Total number of sera from NIF-positive herds; g) % of sera from NIF-positive herd; h) Sera per farm

2.1.2. Examination for the detection of neutralizing antibodies against the BVD-MD virus.

2.1.2.1. Foetal calf kidney (FCK)- cell cultures

2.1.2.1.1. Primary foetal calf kidney cells

Kidneys of 3 to 8-month-old calf foeti, which have been obtained from the slaughterhouse of Hannover, were used. The kidneys were removed from the foeti under sterile conditions and freed of the renal capsule and renal pelvic epithelium as much as possible. The cortex was chopped mechanically and then washed several times with Ca-Mg-free PBS (see appendix 6.2).

The kidney tissue was then trypsinized with a 0.25% solution of trypsin. After stirring for about 10 minutes on a magentic stirrer, the supernatent was decanted and the sediment was further trypsinized for another 60 minutes.

The prepared organ suspension was then filtered through gauze, collected in a centrifuge tube and then centrifuged with a laboratory centrifuge[1] for 10 minutes at 1000 rpm. The cell sediment was then mixed in the ratio of 1:1 with calf serum. For the cultivation of cells, the cell sediment was suspended in the required amount of growth medium (see appendix 6.2), which was adjusted to contain $3x10^5$ cells/ml. The cell suspension was then dispensed in culture bottles, which were afterwards stationarily incubated at 37°C. This was followed by the replacement of the growth medium with a maintenance medium (see appendix 6.2) after two days.

The unrequired cell sediment was mixed with an equal volume of a solution consisting of the growth medium with 10% dimethyl sulfoxide[2], divided into small portions of 2 ml, and then frozen at -70°C (Lindsey and Chow, 1969).

2.1.2.1.2. Secondary and tertiary FCK-cell cultures

In the neutralization test for the detection of BVD-MD antibodies in swine sera using the microtitration method (Frey and Liess, 1971), secondary and tertiary calf kidney cell cultures were released from the glass wall of the culture bottles with a

1) Type UJ KS M. Christ, Osterode

2) E. Merck, Darmstadt.

trypsin-PBS-Versene solution (see appendix 6.2) and the cell suspension was then centrifuged in a laboratory centrifuge[1] at 1200 rpm for 5 minutes.

The cell sediment was then mixed with a maintenance medium and the number of cells adjusted to that number required for the microtitration method (300,000 cells/ml culture medium). The unused cells were employed for the setting up of secondary FCK-cell cultures in culture bottles; the cell concentration was 1-2x10^5 cells/ml of maintenance medium.

When the secondary cell cultures were grown up, they could be also used as tertiary FCK-cells for the neutralization test, after they have been suspended with 0.05% solution of trypsin in PBS-Versene solution (as described above).

2.1.2.2. BVD-MD- challenge virus

2.1.2.2.1. Virus strain

For the detection of neutralizing BVD-MD antibodies in swine sera, the BVD-MD virus strain A 1138/39 (Frey and Liess, 1971) was used.

2.1.2.2.2. Propagation of the virus

Larger quantities of the BVD-MD virus were produced in primary FCK cell cultures. Following the processing of the kidney tissue, the cell suspension was mixed with the growth medium (see appendix 6.2) and then cultivated in 500 ml Meplat's bottles. After being stationarily incubated for 3 days at 37°C, the growth medium was replaced with the maintenance medium (see appendix 6.2) containing 5% foetal calf serum (FCS). This was simultaneously accompanied with the inoculation of the culture bottles, each with 4.5×10^5 to 1.5×10^6 PFU of the BVD-MD virus strain.

After 4 days, the inoculated cultures were kept for 24 hours in a refrigerator at 4°C after the development of the cytopathic effect (CPE) and then subjected to one cycle of freezing and thawing (-70°C, 37°C). The culture fluids were then mixed together, centrifuged in a centrifuge[1] at 3000 rpm for one hour, followed by dispensing the supernatant in 1 ml portions and then kept at -70°C.

1) Type UL 3. M. Christ, Osterod.

2.1.2.2.3. Estimation of the infectivity titer

The infectivity titer of the produced BVD-MD virus was determined by means of the microtitration method, after Frey and Liess (1971).

Tenfold dilutions of the virus were prepared in maintenance medium (see appendix 6.2), and 0.1 ml of each dilution was inoculated in 4 holes of one row of the microtitration plates[1]

0.05 ml of the FCK-cell suspension, previously adjusted to contain 300 000 cells/ml culture medium was then added in the holes using a pipette dropper[2]. The holes of the microtitration plates were covered with air-tight nontoxic adhesive foil[2] and the plates were then incubated at 37 °C. The development of a CPE was daily checked microscopically. The final reading of the results was carried out on the 5th day and the calculation of the virus titer was performed according to Kärber (1931).

2.1.2.3. Microtitration-neutralization test

Each 0.1 ml of the swine sera, previously diluted with the maintenance medium to 1:5 (see appendix 6.2), was put into 4 holes of the first row of the microtitration plates[3] . Moreover, a serum-toxicity control was set up parallely for each serum sample. This was followed by the addition of 0.05 ml of the maintenance medium (see appendix 6.2), using a pipette dropper[2] , into 4 holes of the next 3 rows. Using a calibrated microdiluter of the microtitration Kit[4] 0.05 ml of the serum dilution in the first wellwas transferred to the wells of the next 3 rows, so that 2-fold dilutions from 1:5 to 1:40 developed. In this way, 4 sera could be examined according to this pattern in one microtitration plate. Finally, 0.05 ml of the BVD-MD virus strain A 1138/69, adjusted to contain 50-200 CID_{50}/0.05 ml. was added to each serum dilution using a pipette dropper.

To each of the diluted serum-toxicity control, 0.05 ml of the maintenance medium was added, and the holes of the microtitration plates were sealed with a nontoxic adhesive foil. The incubation was carried out for 2 hours in a refrigerator at -4 °C. After incubation, 0.05 ml cell suspension of the secondary or tertiary

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1) Greiner and Sons, Nürtingen, Württember.
2) Flow Laboratories, Bonn.
3) Greiner and Sons Co., Nürtingen.
4) Flow Laboratory, Bon.

FCK-cells (see 2.1.2.1.2.) was dropped into each hole. This amount of cell suspension was adjusted to contain 300 000 cells/ml of the maintenance medium (see appendix 6.2). The microtitration plates were again covered with adhesive foil and then placed in an incubator at 37 °C.

A cell control was set up parallely with each test row, together with a virus control as well as a virus titration. The reading of the test was carried out on the 3rd, 4th and 5th days.

These sera, which did not reach end point at a dilution of 1:40 were retitrated in another examination series with appropriately higher serum dilution.

2.1.3. Detection of precipitating antibodies against the EHC antigen

2.1.3.1. Virus antigen

The pancreatic glands of EHC-suspicious old sows, from an emergency slaughterhouse, were used for the antigen preparation. The collected pancreases were placed in Petri dishes and transported in cooling boxes to the institute. Where the pancreases were freed from fats, divided into small portions and frozen at -70 °C.

In preliminary trials, the pancreases were tested for their content of precipitinogen against EHC hyperimmune swine sera with the agar-gel diffusion test. The pancreases were then processed according to the purification method of Van Aert (1970), yet with one exception: as no precipitate was obtained by the sedimentation with 30% saturated ammonium sulfate and the subsequent ultra-centrifugation, hence, a 60% saturation was directly used for the precipitation.

All steps were carried out, as much as possible, at 4 °C. Three grams of the reacting pancreatic tissue were homogenized with 12.5 ml of the extraction medium (see appendix 6.2) in a mortar. This step was repeated 3 times. Finally, the obtained pancreatic suspension was stirred for 3 hours with a magnetic stirrer and then ultracentrifuged for 45 minutes at 35 000 g.

The supernatant was mixed with "Frigen-113" in the ratio of 1:3 and then treated for 10 minutes with a laboratory shaker[1]. Afterwards, the aqueous phase of this mixture was centrifuged

1) Turbula, Bachofen Co., Basel.

for 45 minutes at 35 000 g, followed by the dropwise addition of saturated ammonium sulfate solution to the supernatant until a 60% saturation of the supernatant was achieved. This solution was stirred overnight in a magnetic stirrer. After 12-14 hours, the solution was recentrifuged for 45 minutes at 35 000 g.

The obtained sediment was added to 6-fold its volume of extraction medium, divided into small portions of 2 ml each and then kept at -70°C.

2.1.3.2. Control Sera

The EHC-positive swine control serum required for the agar-gel diffusion test consisted of a phenol- containing globulin[1] obtained from EHC hyperimmune serum (Charge No. 510).

The negative control sera consisted of pooled swine sera, which did not reveal the presence of antibodies against the EHC virus in the NIF- test.

2.1.3.3. Microprecipitation

The microprecipitation method[2], after Ouchterlony (1948), was carried out on glass slides covered with a gel layer of 1 mm thickness. Fat-free glass slides were used, placed close to each other in appropriate plastic frames; each frame was adapted for carrying 2 x 3 slides. The frames were fixed on a horizontally leveled table and the slides were then smeared with 0.1% solution of adherent agar (see appendix 6.2). After 15 minutes, 10 ml of heated agar solution (see appendix 6.2) were distributed onto the frame to coat 3 slides.

After being kept for about 30 minutes in a humid chamber, the desired pattern of holes was stamped with the aid of a gel -stamping device which could be moved along the plastic frame. Gel plugs still present in the developed holes were removed by a suction needle fitted to a water-stream suction pump.

Four our examinations we have chosen a ring pattern consisting of one central and 6 peripheral holes. The distance from the six peripheral holes to the central one was 5 mm. After 12-16 hours, he holes were filled up with investigated sera. The agar-gel diffusion test was read under oblique illumination against a black background.

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1) Behringwerke, Marburg/Lahn.

2) LKB Co., Stockholm, Sweden.

The precipitation reactions, which took place at refrigerator temperature, was checked up daily. The first readings were recorded on the 3rd and 4th days. Only those examined sera in which the precipitation line between the antigen and examined sera was blended with the control line between the antigen and hyperimmune sera were evaluated as positive.

2.2. Results

2.2.1. Neutralizing Antibodies Against the BVD-MD Virus

59.5% of the comparable 442 sera from 40 herds, of which 20 revealed negative NIF-results and in the remaining herds one or several NIF-positive sera have been found, revealed the presence of antibodies against the BVD-MD-virus (Table 2) in varying titers (up to 1:640). Table 2 shows, in addition, that the percentage of the BVD-MD positive sera in group A (67.4%) surpassed that of group B (53%) by 14%, but the proportion of the number of herds with or without BVD-MD-positive animals in both groups is nearly similar.

Table 2

Proportion of the number of swine sera with BVD-MD virus neutralizing antibodies to the whole number of tested sera, classified according to the origin of the swine-raising farms with or without simultaneous cattle housing on the same premises.

1	2		3		4		5
Art des a Herkunftsbetriebes[1]	Gesamtzahl b geprüfter		Gesamtzahl e BVD-MD-positiver		Gesamtzahl f BVD-MD-negativer		Prozentsatz g BVD-MD-positiver Seren
	Seren c	Bestände d	Seren	Bestände[2]	Seren	Bestände	
A	276	24	186	22	90	22	67,4
B	166	16	87	15	79	14	53,0
A+B	442	40	273	37	169	36	59,5

1) Farms with (A) or without (B) cattle housing.
2) Herds in which BVD-MD virus-neutralizing antibodies were detected in one or several pigs.

Key:
a) Type of farm of origin; b) Total number of examined; c) Sera; d) Herds; e) Total number of BVD-MD positive; f) Total number of BVD-MD-negative; g) Percentage of BVD-MD-positive sera.

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[illegible], were checked daily. The first readings [illegible]
on the 3rd and 4th days. Only those [illegible]
[illegible] agglutination line between the [illegible]
[illegible] with the control line between the [illegible]
[illegible] sera were evaluated as positive.

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[illegible] NIF-positive sera have been found [illegible]
[illegible] of antibodies against the [illegible]
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[illegible] antibodies [illegible]
[illegible] according to [illegible]
with or without simultaneous [illegible]

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[illegible] with (a) or [illegible] (b) [illegible]
[illegible] and in which [illegible]-positivity [illegible]
[illegible] in one or several [illegible].

[illegible] (a) Total number of [illegible]
[illegible] ND positive; [illegible] Total number of
[illegible] per cent of [illegible]-positive [illegible]

2.2.1.1. Detection of virus-neutralizing antibodies against the BVD-MD virus in all the Sera from NIF-positive herds

As evident from Table 3, the percentage of BVD-MD-positive sera both in group A as well as in group B is somewhat higher than that revealed by the general survey of the serum groups originating from NIF-positive and negative herds, following their examination for the presence of BVD-MD-neutralizing antibodies (Table 2). In Table 3, the percentage of the BVD-MD-positive sera in group A surpassed that of group B by 18.3%. In Table 2, this difference reached 14.4%.

Table 3

Number and percentage of the BVD-MD-positive and negative sera from BVD-MD-positive herds[1)]

1	2	3	4	
Art des (a) Herkunftsbetriebes	Zahl (b) der Bestände	Zahl der (c) vergleichbaren Seren	Zahl der BVD-MD- (d)	
			(e) positiven Seren[2)]	(f) negativen Seren[3)]
A	14	220	163 (74,1%)	57 (25,9%)
B	6	111	62 (55,8%)	49 (44,1%)
A + B	20	331	225 (67,9%)	106 (32,0%)

1) NIF-positive herds are those in which at least one pig reacts positively.
2) Serum titre $\geq$ 1:5
3) Serum titre < 1:5

Key:
a) Type of farm of origin; b) Number of herds; c) Number of comparable sera; d) Number of BVD-MD; e) Positive sera; f) Negative sera.

In Table 3a, in which only NIF-negative swine sera from NIF-positive herds are compared with the BVD-MD-antibodies, the percentage of the BVD-MD-positive reactors of group A (58.6%) is

higher (10.4%) than that of group B (48.2%) though the difference between group A and B is not so great as was represented in the last two tables. Compared with Table 3 (column 4), it is evident that the percentage of the BVD-MD positive sera is about 8% higher than those indicated after the removal of NIF-sera only.

Table 3a

Number and percentage of BVD-MD-positive and negative swine sera among NIF-negative sera from the NIF-positive herds.

1	2	3	4	
Art des Herkunftsbetriebes	Anzahl der Bestände	Anzahl der vergleichbaren Seren	Zahl der BVD-MD- positiven Seren ≧ 1:5	negativen Seren < 1:5
A	14	128	75 (58,6%)	53 (41,4%)
B	6	91	44 (48,2%)	47 (51,8%)
A + B	20	219	119 (54,3%)	100 (45,7%)

Key:
a) Type of farm of origin; b) Number of herds; c) Number of comparable sera; d) Number of BVD-MD; e) Positive sera; f) Negative sera.

It is evident from Table 3a that 106 out of the 112 NIF-positive sera (94.6%) revealed antibodies against the BVD-MD virus. The comparable percentage in Table 3 (67.9%) and Table 3a (54.3%) is essentially lower. The percentage difference of the BVD-MD-positive sera between group A (88 out of 92 sera) and group B (18 out of 20) is only small.

Table 3b:

Number and percentage of BVD-MD-positive and negative swine sera among the NIF-positive swine sera.

1	2	3	4	
Art des Herkunftsbetriebes	Anzahl der Bestände	Anzahl der vergleichbaren Seren	Zahl der BVD-MD- positiven Seren ≧ 1:5	negativen Seren < 1:5
A	14	92	88 (95,6%)	4
B	6	20	18	2
A + B	20	112	106 (94,6%)	6

Key of Table 3b:
a) Type of farm of origin; b) Number of herds; c) Number of comparable sera; d) Number of BVD-MD; e) Positive sera; f) Negative sera.

2.2.1.2. Detection of neutralizing antibodies against the BVD-MD virus in all the sera collected from NIF-negative herds[1]

Table 4 compares the sera from NIF-negative herds. Groups A and B show no essential differences. The percentage of the BVD-MD-positive sera is as a whole (42.3%) less than one half of the comparable percentage (94.6%) of the NIF-positive sera seen in Table 3b. Moreover, if compared with Table 3a (54.3% BVD-MD-positive sera) in which only NIF-negative swine sera from NIF-positive herds are presented, Table 4 reveals a BVD-MD-positive percentage which is 12% lower.

Table 4

Number and percentage of BVD-MD positive and negative swine sera from NIF-negative herds.

1	2	3	4	
Art des (a) Herkunftsbetriebes	Anzahl der Bestände (b)	Anzahl der (c) vergleichbaren Seren	Zahl der BVD-MD- (d) positiven Seren (e) ≧ 1:5	negativen Seren (f) <1:5
A	10	56	23	33
B	10	55	25	30
A + B	20	111	48 (42,3%)	63 (57,7%)

Key:
a) Type of farm of origin; b) Number of herds; c) Number of comparable sera; d) Number of BVD-MD; e) Positive sera; f) Negative sera.

1) See footnote of Table 3.

2.2.1.3. Numerical Proportion of EHC and BVD-MD virus-neutralizing sera in NIF-positive herds.

Table 5 indicates that 18 out of the 20 examined NIF-positive herds showed more BVD-MD-positive than NIF-positive sera, with the exception of one herd in which the number of NIF-positive sera, exceeded (herd Gro., see 6.1.)., whereas only 2 out of 19 sera inhibited te EHC-virus in a dilution of 1:5, and none of the 19 sera revealed the presence of BVD-MD virus-neutralizing antibodies.

Table 5

Number of NIF-positive herds classified according to the numerical proportion between the NIF-positive and BVD-MD-positive sera

Zahlenverhältnis 1)	Gruppe A 2)	Gruppe B 2)	Gruppe A + B 2)
NIF-positive Seren / BVD-MD-positive Seren 3) > 1	1 Bestand 4)	0	1 Bestand 4)
NIF-positive Seren / BVD-MD-positive Seren < 1	12 Bestände 5)	6 Bestände 5)	18 Bestände 5)
NIF-positive Seren / BVD-MD-positive Seren = 1	1 Bestand 4)	0	1 Bestand 4)

Key:
1) Numerical porportion; 2) Group; 3) NIF-positive sera / BVD-MD-positive sera ;
4) Herd; 5) Herds.

2.2.1.4. Comparison of the neutralizing titers against EHC and BVD-MD virus.

From a total of 328 swine sera from NIF-positive herds, 101 sera (30.8%) revealed no neutralizing antibody in the serum dilution of < 1:5 against the EHC and BVD-MD viruses. Only six sera neutralized the EHC virus without being able to neutralize the BVD-MD virus at the same time. On the other hand, 116 sera showed a neutralizing activity against the BVD-MD virus, but they were also unable to neutralize the EHC virus. Only 105 out of the 328 sera (32%) neutralized the EHC virus as well as the BVD-MD virus (Table 6). It is obvious (Fig. 1) that the number of swine

sera, in which neutralizing antibodies against the EHC virus were detected, decreased gradually with the increase of the serum dilution. On the contrary, the number of sera with neutralizing antibodies against the BVD-MD virus increased with the increase of the serum dilution till 1:40 and then it dropped. The number of sera with an SN_{50} of 1:160/1:320 was somewhat higher and then dropped entirely at 1:640.

Table 6

Results of the examination of 328 swine sera from NIF-positive herds with reference to the frequency of the detection of neutralizing antibodies in the titer ranges between 1:5 and 1:640

a) Neutralisationstiter[1] gegen BVD-MD-Virus

b) Neutralisationstiter gegen ESP-Virus	< 5	5	5-10	10-20	20-40	40-80	80-160	160-320	320-640	
< 5	101	1	19	33	29	5	9	14	6	217
5	3	-	2	5	8	3	6	4	2	33
5-10	1	-	-	9	8	2	5	7	1	33
10-20	-	1	2	4	10	3	2	4	-	26
20-40	-	-	-	1	3	1	3	4	-	12
40-80	1	-	2	-	-	-	1	-	-	4
80-160	1	-	-	1	-	-	-	-	-	2
160-320	-	-	-	1	-	-	-	-	-	1
	107	2	25	54	58	14	26	33	9	328

1) Reciprocal value of the SN_{50} betwenn $\log_2$-serum dilutions.

Key:
a) Neutralization titer*) against the BVD-MD virus; b) Neutralization titer*) against the EHC virus.

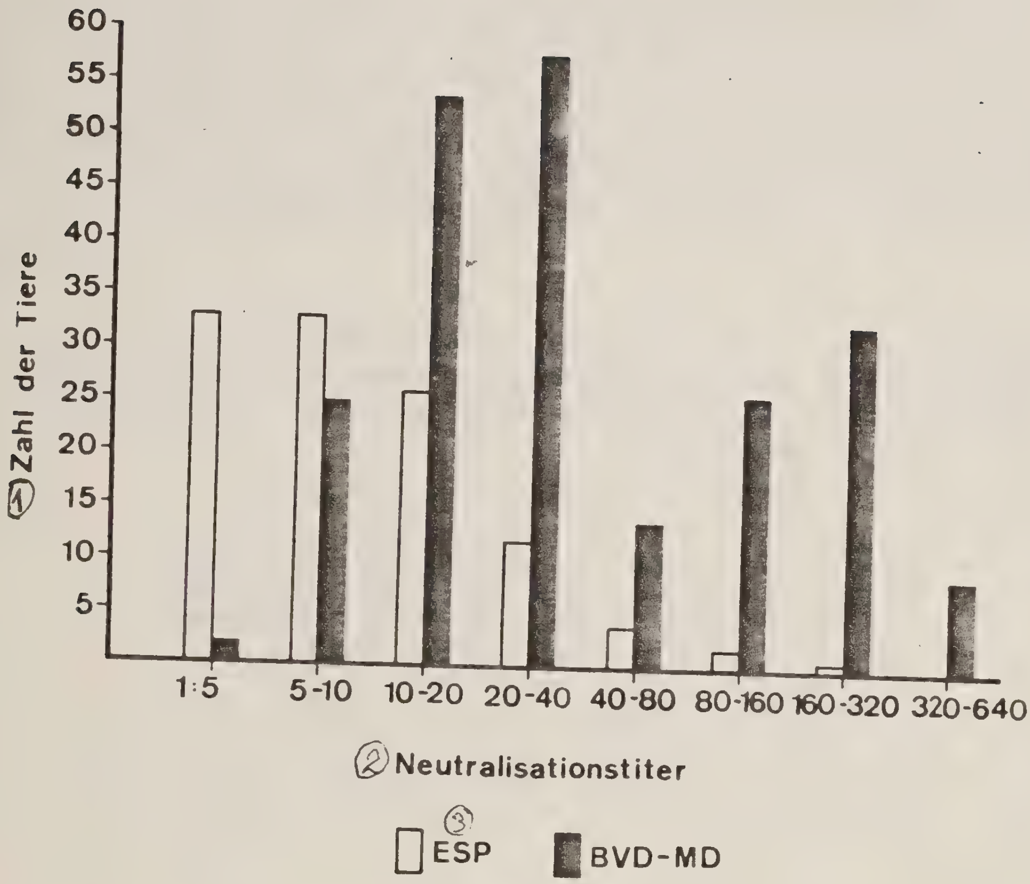

Fig. 1. Number of the animals from NIF-positive herds with neutralization titers $\geq$ 1:5 against EHC and BVD-MD viruses.

Key:
1) Number of animals; 2) Neutralization titer; 3) EHC.

2.2.2. Microprecipitation test with an EHC antigen prepared from swine pancreas, after Van Aert.

2.2.2.1. Frequency of the precipitation reactions in all the tested swine sera

Table 7 summarizes the results of the microprecipitation tests with reference to the whole number of tested swine sera classified according to the origin of the swine-raising farms with or without

cattle housing on the same premises. The whole percentage of sera with EHC-precipitating antibodies of 43.1% (Table 7) was higher than the percentage of NIF-positive sera of 25.4% (112 out of 442 sera), but lower than that of the BVD-MD-neutralizing sera of 59.5% (Table 2). The high difference (about 20%) between the sera of group A (farms with cattle housing) and group B (only swine raising) is conspicuous which is evident in Table 7.

Table 7

Proportion of the number of swine sera with precipitation reaction to the whole number of tested sera, classified according to their origin either from swine-raising farms (A) or those without cattle housing (B)

1	2		3		4
Art des (a) Herkunftsbetriebes	Gesamtzahl geprüfter (b)		(e) Gesamtzahl der Präzipitationen		(f) Prozentsatz präzipitierender Seren
	(c) Seren	Bestände (d)	(c) Seren	(d) Bestände	
A	271	24	138	21	50,9
B	168	16	51	13	30,4
A + B	439	40	189	34	43,1

Key:
a) Type of farm of origin; b) Total number of tested; c) Sera; d) Herds; e) Whole number of precipitation reaction; f) Percentage of precipitating sera.

2.2.2.2. Comparison of the precipitation and NIF-results

Table 8 presents the frequency of the precipitation reactions with swine sera from NIF-positive herds without making a classification of the NIF-positive and the NIF-negative sera (Tables 8a and 8b). The percentage of precipitating sera (43.1%) has not changed as compared with the results of the whole number of sera (Table 7). But a considerable shifting has resulted between groups A and B as the difference increased to 33.2%. After the classification of all sera from the NIF-positive herds (see footnote of Table 3)

into NIF-negative and NIF-positive sera and their comparative examination for the frequency of precipitation reactions, the percentages shown in Tables 8a and 8b resulted. In this regard, the higher percentages of precipitation reactions among the NIF-positive sera became conspicuous and a considerable difference (39.8%) existed between the sera of groups A and B among the NIF-negative sera (Table 8a) in contrast to the relatively equal comparison figures among the NIF-positive sera (Table 8b).

Table 8

Frequency of the precipitation results with the NIF-results

1	2	3	4	
Art [1] des Herkunftsbetriebes a	Zahl der Bestände b	Zahl der vergleichbaren Seren c	Präzipitierende Seren d	
			Zahl e	%
A	13	215	119	55,3
B	6	113	25	22,1
A + B	19	328	144	43,9

1) see Table 7.

Key:
a) Type of farm of origin; b) Number of the herds; c) Number of comparable sera; d) Precipitating sera; e) Number.

Table 8a

Frequency of the precipitation reactions among the NIF-negative sera from NIF-positive herds

1	2	3	4	
Art des Herkunftsbetriebes a	Zahl der Bestände b	Zahl der vergleichbaren Seren c	Präzipitierende Seren d	
			Zahl e	%
A	13	129	71	55
B	6	92	14	15,2
A + B	19	221	85	38,5

Key:
a) Type of farm of origin; b) Number of the herds; c) Number of comparable sera; d) Precipitating sera; e) Number.

Table 8b

Frequency of the precipitation reactions among NIF-positive sera

1	2	3	4	
Art des Herkunftsbetriebes (a)	Zahl der Bestände (b)	Zahl der vergleichbaren Seren (c)	Präzipitierende Seren (d)	
			Zahl (e)	%
A	13	86	48	(55,8)
B	6	21	11	(52,4)
A + B	19	107	59	55,1

Key:
a) Type of farm of origin; b) Number of the herds; c) Number of comparable sera; d) Precipitating sera; e) Number.

Table 9

Frequency of the precipitation reactions with swine sera from NIF-negative herds (see Table 8b)

1	2	3	4	
Art des Herkunftsbetriebes (a)	Zahl der Bestände (b)	Zahl der vergleichbaren Seren (c)	Präzipitierende Seren (d)	
			Zahl (e)	%
A	10	56	19	(33,9)
B	10	55	26	(47,3)
A + B	20	111	45	40,5

Key:
a) Type of farm of origin; b) Number of the herds; c) Number of comparable sera; d) Precipitating sera; e) Number.

Table 9 presents a comparison between the whole percentage of precipitating sera (swine sera from NIF-negative herds) and that of Table 8b (NIF-positive sera). The difference is only 15% A comparison of the percentage of groups A and B is not possible as the number of sera is small ($<$100). The whole percentage of precipitating sera (40.5%), indicated in Table 9, is remarkably equal to that of the NIF-negative sera from NIF-positive herds (Table 8a). This statement suggests a comparison with the results of the BVD-MD- neutralization test.

3. Discussion of the Results

The frequency of swine sera with BVD-MD virus neutralizing antibodies in an unarranged group of sera comprising 442 samples of breeding pigs from 40 piglet rearing farms was all of a sudden high (Table 2: 59.5%). But this statement does not help in drawing any conclusion on the general situation prevailing in the swine population, because among the 40 piglet- rearing farms, 20 were designated as "NIF-positive" herds, according to a previous examination for the presence of neutralizing antibodies against the EHC virus (NIF-test). The remaining 20 herds were NIF-negative; nevertheless, serum samples were not available from all animals, but only from an average of 39% of the breeding animals per herd in contrast to the almost 100% of animals from the NIF-positive herds. However, the correlation between the BVD-MD and EHC antibodies is still in question. The results of the examination of swine sera collected from swine herds, rather than experimentally infected animals, are known to a very limited extent. The informations given by Snowdon and French (1968) are only confined to the sera of 70 pigs which revealed the presence of BVD-MD or EHC-neutralizing antibodies either alone or in combination with EHC-precipitating antibodies (60 sera). From these 60 swine sera, 85% (= 51 sera: 72% of the total number) had BVD-MD antibodies. Moreover, Stewart et al., (1971) referred to the results obtained from swine herds in which most of the pigs revealed BVD-MD neutralizing antibodies (17 out of 25) and a smaller number (10 out of 17) had very small antibody titers against the EHC virus following the vaccination of cattle against BVD-MD or the feeding of offals from cattle. These results cannot be compared with those reported in the present work and they can only prove the expectation of a high number of BVD-MD neutralizing sera in the swine population. Finally, these results led to the conduction of some examinations in order to determine whether the BVD-MD virus can infect pigs, and induce the production of antibodies (Snowdon and French, 1968; Stewart et al., 1971; Phillip and Darbyshire, 1972).

With this understanding, it was suggested to check up whether the classification of all the blood sera (investigated for the presence of BVD-MD antibodies) into farms with cattle housing on the same premises and those raising pigs only will lead to the detection of a difference in the BVD-MD- antibodies. In this regard, the result of the examination of sera from NIF-positive herds, in which the number of the BVD-MD-positive sera in group A (farms with cattle-housing) were always higher than that of the group B (Table 3 and the following tables), is remarkable. On the other hand- even when the number of sera was small- according to the results of Table 4, that the percentage of the BVD-MD-positive sera of groups A and B was equal, and in relation to the whole number of these 111 sera, nearly 42% reacted positively for the presence of BVD-MD. The majority of these sera revealed antibody titers between 1:10 and 1:40 and a titer of $\geq$ 1:80 was obtained only by 10 sera. Comparatively, the majority of antibody titers of the sera from NIF-positive herds were between 1:20 and 1:40 and it is noteworthy to mention here that the number of sera with titers of $\geqslant$ 1:80-1:320 increased again (Fig. 1), a fact particularly remarkable in group A in which there was a possibility for the frequent infection and "booster effect" through the mixed swine- cattle rearing. The correlation between the NIF-reactions and the detection of BVD-MD antibodies is obvious from Table 6 (distribution of titers). Accordingly, it could be proved that the antibody titers against the BVD-MD virus in the NIF- and BVD-MD-positive sera were, as a rule, clearly higher than those against the EHC virus, where a difference of $\log_2$-dilution was not interpreted while considering the different titration systems. Nevertheless, as indicated in the individual survey of the sera presented in the appendix, no general or common correlation can be established between the high BVD-MD and the low EHC antibody titers. The examples presented by the herds Wi., Ge., and Scho specially supported this statement. It is clear from these examples that antibodies against the EHC virus do not necessarily bring on antibodies against the BVD-MD virus, whereas the reverse is frequently the case. It can be just as little proved that the high antibody titers against the EHC virus and the low titers against the BVD-MD virus rule out the reversible correlations within a certain herd (herd Wi.). In this connection, it should be taken into consideration that in the NIF-positive herds as well, 42.3% of the animals revealed the presence of BVD-MD antibodies (Table 4). Out of the 31 serum samples from the herd Wi., which could be evaluated, 13 (about 42%) sera were BVD-MD-positive: the standard portion beyond which an additional EHC infection independent of the BVD-MD will lead to the additional production of antibodies. Further examinations must be

This study [illegible]
tration of all the blood [illegible]
of BVD-V antibodies [illegible]
remaining and those [illegible]
a difference in the BVD-MD [illegible]
the examination [illegible]
sum [illegible] of the BVD-MD [illegible]
[illegible] were always high [illegible]
the following [illegible]
the number of sera was [illegible]
that the percentage [illegible]
equal, [illegible] in relation [illegible]
reacted positively for [illegible]
there were several [illegible]
a [illegible] was [illegible]
ly, the ma[illegible]
the herds were [illegible]
here that [illegible]
and again (Fig. [illegible])
it was a possibility [illegible]
effect [illegible]
Both [illegible]
is obvious. From Table [illegible]
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titers in the [illegible]
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these samples [illegible]
essentially [illegible]
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observed that [illegible] high [illegible]
the low titers [illegible]
correlation within [illegible]
it should be [illegible]
herds as well [illegible] (Table [illegible]).
VD-MD [illegible]
the herd [illegible]
MD-positive [illegible]
intensive [illegible]
small size. [illegible]

carried out to answer this question. Anyway, the results of the present work do not seem to exclusively support those of Snowdon and French (1968) and those of Stewart et al., (1971), according to which the EHC neutralizing antibodies can be interpreted as a sequence of the detectable too high antibody titers of the widely distributed BVD-MD antibodies. Further investigations were also necessary in order to find out the influence of the standard portion of BVD-MD antibodies relative to the antibody titers upon the epizootiological course of EHC in a certain swine population and upon the clinical course of illness in individual animals. In such a way, it might be possible to tackle the problem of establishing subclinically latent farms of EHC. It is first of all necessary to determine the standard portion of BVD-MD antibodies among pigs by examining a large group of sera from NIF-negative herds; this will be also important for the interpretation of the second part of the present examinations, which deals with the results of the micro-precipitation tests done on the same groups of sera.

The technique of the microprecipitation test had the great advantage of requiring only small amounts of the reagents and particularly the tested sera, which were either available in small amounts or completely exhausted so that, in some cases, no additional examination could be carried out. Moreover, the precipitation reactions were well readable without requiring a previous shaking of the sera with chloroform (Woernle, 1961). Weak and strong reactions were recognizable, yet, the interpretation was carried out only qualitatively. On this basis, the frequency of the precipitation reactions in the individual subgroups of sera was compared with that of the NIF- and BVD-MD reactions.

The high percentage of precipitating sera in the samples of swine serum from NIF-negative herds (Table 8b), which with 40.5% lay within the range of frequency of the BVD-MD-positive sera (see Table 4: 42.3%), was remarkable. On the other hand, the slight coincidence of 55.1% (Table 8b) with 94.6% BVD-MD-positive and NIF-positive sera (Table 3b) was also observed. Finally, it was also remarkable that precipitation reactions among sera from swine herds with which cattle were raised and the BVD-MD antibodies in the NIF-herds appear more frequently than in those swine herds without simultaneous cattle-housing (Table 8). Although— by using the method of detecting precipitating antibodies against a soluble antigen— a smaller differentiation ability between BVD-MD and EHC virus antigens was expected than that achieved by using the neutralization test, yet the individual surveys (see appendix 6.1.) and especially those of the herds Wi., Ge. and Scho. should be taken into consideration as was already mentioned regarding the

BVD-MD reactions. As evident from such individual surveys, precipitations occurred mainly with those EHC sera of high titers (herd Wi.), but not with those of lower EHC titers ($\leq$ 1:64), although they also occurred in case of sera with neither BVD-MD nor EHC neutralizing antibodies. On the contrary, the micro-precipitation test seems to include BVD-MD sera of higher titers only, and occasionally also sera with lower titers (a fact which cannot be explained). The objectivation and interpretation of the results are confronted with difficulties and require the referring to individual combinations of NIF-, BVD-MD and EHC precipitation reactions (see appendix 6.1). Furthermore, according to the only moderate value of such comparisons of result combinations, and particularly by considering the small numbers of sera examined, a trend can be perhaps represented as will be evident from the present case. Apart from the combinations:

NIF-positive/BVD-MD-positive/EHC-precipitation positive (21.3%) and

NIF-negative/BVD-MD-negative/EHC-precipitation negative (13.8%), which, together, amount to 35.1% of the whole number of sera constituting group A (swine-cattle housing), the combinations

a) NIF-positive/BVD-MD-positive/EHC-precipitation positive (21.8%).

b) NIF-positive/BVD-MD-negative/EHC-precipitation negative (0.9%) (together 22.7%)

and

c) NIF-positive/BVD-MD-negative/EHC-precipitation positive (12.8%)

d) NIF-negative/BVD-MD-positive/EHC-precipitation negative (0.9%) (together 13.7%)

must be of interest in order to check up the coincidence of the precipitation reaction with the BVD-MD results (a+b) in comparison with the coinciding NIF- and precipitation results (c+d). As the frequency of the combinations (a+b) is considerably higher than that of (b+c), hence, it can be at least concluded that the soluble EHC antigen reacts group-specifically with the BVD-MD antibodies. Accordingly, the combination (a) represents the so-called "false-positive EHC-, true positive BVD-MD reactions", while in case of the not yet mentioned combination:

e) NIF-negative/BVD-MD-negative/EHC-precipitation positive, which appeared 30 times among 321 sera from NIF-positive herds (= 9.3%) and 29 times among 111 sera from NIF-negative herds (= 26.1%), such a correlation is not permissible. This does not, however, mean that it is a matter of specific reactions— which

have nothing to do with EHC infections— that can frequently lead to errors by the serological diagnosis of the EHC than the NIF test. Concerning the NIF test for detecting the EHC neutralizing antibodies and not the BVD-MD neutralizing ones, all these NIF-positive sera and their frequency had to be determined in the individual comparison, according to which it can be assumed, based upon the higher BVD-MD antibody titers ($> \log_2$-dilution), that they are only BVD-MD sera; this seems to be the case with 91% of the 115 positive sera (79% BVD-MD-positive sera). On the contrary, and on the same basis of interpretation, 56.6% of the precipitation reactions have correlated with the BVD-MD-positive reactions (115 out of 203) among those sera which reacted only positively for BVD-MD- or which revealed BVD-MD antibodies with a titer higher than that of the antibodies against the EHC virus with more than $\geq \log_2$-dilution, i.e. less than between NIF-positive and BVD-MD-positive sera. This result is not surprizing, because by applying the serum neutralization test finer antigenic differences could be determined than in the case of the precipitation reaction. However, when we consider the 91 NIF-positive BVD-MD sera relative to the whole number of "true" BVD-MD-positive sera (203), the NIF- test will show a lower "false-positive" reaction quota (44.8%) than that obtained in the precipitation test. If the selection of the EHC-suspicious herds was done depending upon the precipitation reaction carried out on random samples, their, 15 out of 20 NIF-positive herds would be found positive which necessitates the additional application of the NIF test and the BVD-MD neutralization test.

Based upon the comparative serological examinations, the herd of the farm Wi. is considered a suspicious-free EHC herd. Regarding the herd Ge., the EHC- and BVD-MD titers of some individual sera were almost of the same strength; but in other herds, the EHC titers were equal to or even higher than the BVD-MD titers (herds Eu., Vo., and Scho.), so that the question of whether sera of these herds— which have partly shown high titers of BVD-MD antibodies— were the result of subclinical EHC infectious that could be serologically weak but immunologically well tolerated, seems to be justified.

This possibility has been pointed out in the experimental results of Sheffy et al. (1962), according to which the infection of pigs with the BVD-MD virus (strain Oregon $C_{24}V$) done for the first time led to the induction of only BVD-MD virus-neutralizing antibodies and the reinfection with EHC virus carried out 4 weeks later led to a subsequent rise of the titer. However, this was accompanied by the induction of lower titers of EHC virus-neutralizing antibodies.

Finally, depending upon the described results of the present work, the question mentioned in the introduction concerning the suitability of the agar-gel precipitation test in the serological screening tests for detecting the EHC antibodies in comparison with the method of detecting EHC virus-neutralizing antibodies can be only negatively answered.

4. Summary

1. Out of 457 pig sera, previously tested for antibodies against hog cholera virus with the aid of the NIF test, 442 could be screened for antibodies against BVD-MD virus. In 273 sera (59.5%) neutralizing antibodies could be detected and were shown to possess titers between 1:5 and 1:640.

2. The number of BVD-MD virus-neutralizing sera from NIF-positive herds (one or more hog cholera virus-neutralizing sera per herd) was 225 (67.9%) compared with 48 (42.3%) from NIF-negative herds.

3. Of 112 sera with hog cholera virus-neutralizing antibodies (NIF-positive sera) 106 (94.6%) also neutralized BVD-MD virus, whereas out of 219 NIF-negative sera from NIF-positive herds only 119 (54.3%) showed BVD-MD virus-neutralizing activity.

4. The frequency of BVD-MD virus-neutralizing sera required differentiation between pig herds with (group A) and without cattle housing on the same premises (group B), in order to detect interrelationships between pigs and cattle with respect to the prevalence of BVD-MD virus-neutralizing antibodies. In group A (276 sera from 24 hers) the number of BVD-MD virus-neutralizing sera surpassed with 186 (67.4%) those of group B (166 sera from 16 herds) by 15.4%.

5. Evaluation of titers of neutralizing antibodies against hog cholera and BVD-MD virus showed a continuous decrease of the number of hog cholera virus-neutralizing sera with increasing dilution. The number of BVD-MD virus-neutralizing sera, however, increased continuously from 1:5 up to 1:20/40 and after a distinct reduction at 1:40/80 rose again to 1:160/320. Nine sera revealed antibody titers of 1:640.

6. In 189 sera (43.1%) out of 439 precipitating antibodies could be demonstrated against a hog cholera antigen produced from porcine pancreas by extraction according to the method of van Aert.

7. From NIF-positive herds 43.9% of the sera reacted in the agar-gel double diffusion test, from NIF-negative herds 40.5%. Of the NIF-positive sera 55.1% showed a precipitation line, whereas 38.5% of the NIF-negative sera from NIF-positive herds gave positive precipitation. Sera from NIF-positive herds with cattle (group A) precipitated to a higher percentage (55.3%) than sera from NIF-positive herds without cattle (22.1%).

8. 93% of the sera which possessed neither hog cholera nor BVD-MD virus-neutralizing activity gave positive precipitation. Most of the precipitation reactions could be detected with sera in which BVD-MD and/or hog cholera virus-neutralizing antibodies could be demonstrated. Individual comparisons of the precipitating sera resulted in the finding of higher correlation to BVD-MD-positive (21.8%) than to hog cholera positive sera (12.8%).

9. Further comparisons of the serological results led to the conclusion that the hog cholera antigen extracted from pig pancreatic tissue reacted group specifically with BVD-MD antibodies. Therefore the agar-gel double diffusion test seemed less suitable for studies on the occurrence of hog cholera- induced antibodies than the demonstration of virus-neutralizing antibodies (NIF-test).

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Appendices

Individual Review of the Results of Serological Examinations

I. NIF-positive swine herds

A. Herds with cattle housing on the same premises

Result of Examination

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP- 1 Präzipitationstest
		2 Bestand Ec.	
1871	<1:5	<1:5	+
1872	<1:5	n.u.	n.u. 3
1873	<1:5	1:80	-
1874	<1:5	1:30	-
1875	1:5	1:160	-
1876	<1:5	1:160	+
1877	<1:5	<1:5	-
1878	<1:5	<1:5	-
		2 Bestand Pa.	
823	1:8	>1:160	n.u. 3
824	<1:5	1:20	-
825	<1:5	<1:5	-
826	1:16	1:160	-
827	1:32	1:40	-
828	<1:5	<1:5	- 3
829	1:8	>1:160	n.u.
830	<1:5	<1:5	- 3
831	1:8	1:40	n.u.
832	<1:5	1:40	+
833	<1:5	1:30	-
834	<1:5	1:15	-
835	<1:5	1:10	-
836	<1:5	1:20	-
837	1:64	>1:40	-

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP-① Präzipitationstest
		② Bestand N. & Ge.	
863	1:16	1:80	-
864	1:5	1:160	n.u.③
865	<1:5	<1:5	-
866	<1:5	1:240	-
867	1:16	1:30	-
868	1:8	1:20	n.u.③
869	1:5	1:20	-
870	<1:5	<1:5	-
871	1:32	1:60	n.u.③
872	1:8	1:160	n.u.③
		② Bestand Eg.	
605	<1:5	1:20	-
606	<1:5	1:40	+
607	1:16	1:30	+
608	1:16	1:30	-
609	1:16	1:30	-
1412	1:8	1:30	-
1413	<1:5	<1:5	-
1414	<1:5	<1:5	-
1415	<1:5	1:320	-
1416	<1:5	<1:5	-
1417	1:16	1:30	-
1418	<1:5	<1:5	-
1419	<1:5	<1:5	-
1420	<1:5	<1:5	-
		② Bestand Gra.	
535	1:5	1:480	-
536	1:5	1:40	-
537	1:20	1:40	-
1821	<1:5	1:30	-
1822	<1:5	<1:5	+
1823	<1:5	1:15	-
1824	<1:5	1:20	+
1825	<1:5	1:30	+
1826	<1:5	<1:5	-

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP-① Präzipitationstest
		② Bestand Eu.	
2199	<1:5	1:15	+
2200	1:10	1:20	-
2201	<1:5	1:30	-
2202	<1:5	1:30	+
2203	1:10	1:15	+
2204	<1:5	1:15	-
2205	<1:5	1:15	-
2206	<1:5	1:15	-
2207	1:20	1:7,5	+
2208	1:5	1:15	+
2209	1:20	1:30	+
2210	<1:5	<1:5	-
2211	<1:5	1:30	+
2212	<1:5	1:30	+
2213	<1:5	1:15	+
2214	1:20	1:15	+
2215	1:10	1:15	+
2216	<1:5	1:7,5	-
2217	<1:5	<1:5	+
2218	1:20	1:15	+
2219	1:10	1:15	+
2220	1:10	1:15	+
		② Bestand Ha.	
1359	1:5	1:160	+
1360	<1:5	<1:5	+
1361	<1:5	1:40	+
2250	<1:5	<1:5	+
2251	<1:5	<1:5	+
2252	<1:5	1:10	+
2253	<1:5	1:15	+
2254	<1:5	1:7,5	+

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP- ① Präzipitationstest
		② Bestand Vr.	
1362	< 1:5	1:30	-
1363	1:5	1:15	-
1364	1:5	1:40	+
1365	< 1:5	1:640	+
1366	1:20	1:80	-
2236	< 1:5	1:20	-
2237	< 1:5	< 1:5	+
2238	< 1:5	< 1:5	+
2239	< 1:5	n.u. 3	+
2240	1:20	n.u.	-
2241	< 1:5	1:20	+
2242	1:20	n.u.	+
2243	< 1:5	1:7,5	+
2244	< 1:5	1:15	-
2245	< 1:5	< 1:5	+
2246	< 1:5	1:10	+
2247	< 1:5	< 1:5	+
2248	< 1:5	1:7,5	+
2249	< 1:5	< 1:5	+
		② Bestand Gro.	
1403	< 1:5	< 1:5	-
1404	< 1:5	< 1:5	-
1405	< 1:5	< 1:5	-
1406	1:5	< 1:5	+
1407	1:5	< 1:5	-
2294	< 1:5	< 1:5	+
2295	< 1:5	< 1:5	-
2296	< 1:5	< 1:5	-
2297	< 1:5	< 1:5	+
2298	< 1:5	< 1:5	+
2299	< 1:5	< 1:5	+
2300	< 1:5	< 1:5	+
2301	< 1:5	< 1:5	-
2302	< 1:5	< 1:5	+

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP- (1) Präzipitationstest
2303	<1:5	<1:5	-
2304	<1:5	<1:5	+
2305	<1:5	<1:5	-
2306	<1:5	<1:5	-
2307	<1:5	<1:5	+
		(2) Bestand Vo.	
2270	1:5	1:80	+
2271	1:40	1:160	-
2272	1:40	1:320	+
2273	1:20	1:10	+
2274	<1:5	n.u.	n.u. (3)
2275	1:5	1:10	+
2276	1:10	1:40	n.u. (3)
2277	1:10	1:320	+
2278	1:40	1:160	+
2279	<1:5	1:30	+
2280	<1:5	1:15	+
2281	<1:5	1:40	+
2282	1:20	1:40	+
2283	1:10	1:30	+
2284	1:5	1:40	+
		(2) Bestand Schü.	
2255	1:5	1:240	+
2256	<1:5	1:320	+
2257	1:20	1:80	+
2258	1:20	1:320	+
2259	1:20	1:240	-
2260	1:10	1:40	+
2261	1:40	1:30	+
2262	<1:5	1:320	+
2263	1:10	1:40	+
2264	1:20	1:5	+
2265	<1:5	1:320	+
2266	1:10	1:80	+

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP-Präzipitationstest [1]
2267	1:40	1:160	+
2268	1:20	1:240	+
2269	1:10	1:40	+
		Bestand Re. [2]	
1703	1:40	1:320	-
1704	1:40	1:320	-
1705	1:10	1:240	-
1706	1:5	1:240	-
1707	1:40	1:320	-
1708	1:20	1:320	-
2285	1:5	1:20	+
2286	<1:5	1:240	+
2287	1:10	1:240	+
2288	1:5	1:160	+
2289	<1:5	<1:5	+
2290	<1:5	<1:5	+
2291	1:5	<1:5	+
2292	1:5	1:40	n.u. [3]
2293	<1:5	<1:5	+
		Bestand Kü. [2]	
1735	1:5	<1:5	-
1736	1:10	1:160	-
1737	1:20	1:40	-
1738	1:10	<1:5	-
1739	1:10	1:15	-
2221	<1:5	1:10	-
2222	1:5	1:80	-
2224	<1:5	1:15	-
2225	1:10	1:160	+
2226	<1:5	1:20	+
2227	<1:5	1:7,5	-
2228	<1:5	1:7,5	-
2229	<1:5	1:15	-
2230	1:20	n.u.	-
2223	<1:5	1:7,5	-

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP-Präzipitationstest (1)
2231	<1:5	1:20	+
2232	<1:5	1:7,5	+
2233	<1:5	<1:5	-
2234	<1:5	<1:5	-
2235	<1:5	1:40	-
		(2) Bestand Br.	
4408	<1:5	1:40	+
4409	<1:5	<1:5	+
4410	<1:5	1:40	+
4411	<1:5	1:640	+
4412	<1:5	<1:5	-
4413	<1:5	<1:5	+
4414	<1:5	<1:5	+
4415	1:5	1:15	+
4416	1:5	1:40	+
4417	1:10	1:120	+
4418	<1:5	1:160	+
4419	1:5	1:160	+
4420	1:5	1:240	+
4421	1:10	1:40	-
4422	<1:5	1:120	+
4423	<1:5	1:320	+
4424	<1:5	1:80	-
4425	<1:5	1:240	+
4426	<1:5	1:20	+
4427	1:10	1:320	+
4428	1:5	1:160	-
4429	1:5	1:240	-
4430	1:10	1:640	-
4431	1:5	1:30	+
4432	<1:5	1:160	+
4433	<1:5	1:640	-
4434	1:5	1:80	-
4435	<1:5	1:80	+
4436	<1:5	1:20	+
4437	<1:5	1:320	+

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP- 1) Präzipitationstest
4438	<1:5	1:40	+
4439	<1:5	1:120	+
4440	<1:5	1:80	-
4441	<1:5	1:640	+
4442	1:5	1:640	+
4443	1:10	1:320	+
4444	<1:5	1:20	+

Key:
1) EHC-precipitation test; 2) Herd; 3) Not examined.

B. Herds without cattle housing on the same premises

Result of Examination

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP- 1) Präzipitationstest
		2) Bestand St.	
1897	<1:5	1:20	-
1898	<1:5	1:40	-
1899	<1:5	1:30	-
1900	<1:5	<1:5	-
1901	1:5[1)]	1:40	-
1902	<1:5	1:15	-
1903	<1:5	1:40	-
1904	<1:5	1:10	-
1905	<1:5	1:320	-
1906	<1:5	<1:5	-
1907	<1:5	1:5	-
1908	<1:5	1:640	+
1909	<1:5	1:160	-
1910	<1:5	1:7,5	+
1911	<1:5	1:20	-
1912	<1:5	1:160	-
1913	<1:5	<1:5	+

1) A second examination gave a titer of < 1:5

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP-Präzipitationstest [1]
	[2] Bestand Wi.		
911	1:128	<1:5	+
912	<1:5	<1:5	+
913	<1:5	<1:5	+
914	<1:5	<1:5	-
915	<1:5	<1:5	-
916	1:8	1:80	-
917	<1:5	1:30	+
918	1:128	n.u.	n.u. [3]
919	1:128	1:15	+
920	<1:5	<1:5	-
921	1:16	1:30	+
922	<1:5	<1:5	+
923	1:128	n.u.	+
924	1:64	<1:5	+
925	1:16	1:160	n.u. [3]
926	1:64	1:7,5	+
927	1:32	1:30	+
1879	<1:5	<1:5	-
1880	<1:5	<1:5	-
1881	<1:5	<1:5	-
1882	<1:5	<1:5	-
1883	<1:5	1:7,5	-
1884	<1:5	1:7,5	+
1885	<1:5	1:7,5	-
1886	<1:5	<1:5	+
1887	<1:5	n.u.	n.u. [3]
1888	<1:5	n.u.	n.u. [3]
1889	<1:5	1:5	-
1890	<1:5	1:15	-
1891	<1:5	<1:5	-
1892	1:64	1:7,5	-
1893	<1:5	<1:5	-
1894	<1:5	<1:5	-
1895	1:256	1:20	+
1896	<1:5	<1:5	-

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP-Präzipitationstest 1
	2 Bestand Grü.		
375	1:8	1:320	+
376	1:5	1:30	-
377	1:8	1:20	-
378	1:5	1:10	-
379	<1:5	1:10	-
	2 Bestand Ge.		
812	<1:5	<1:5	+
813	<1:5	<1:5	-
814	<1:5	<1:5	-
815	<1:5	<1:5	-
816	<1:5	<1:5	-
817	1:32	1:20	-
818	<1:5	n.u.	-
819	1:32	n.u.	n.u. 3
820	1:16	1:15	-
821	<1:5	<1:5	-
822	1:16	1:15	-
1930	<1:5	<1:5	-
1931	<1:5	<1:5	-
1932	<1:5	<1:5	-
1933	<1:5	1:15	-
1934	<1:5	1:160	-
1935	<1:5	<1:5	-
1936	<1:5	1:320	-
1937	<1:5	<1:5	-
1940	<1:5	<1:5	-
1941	<1:5	<1:5	-
1942	<1:5	<1:5	-
1943	1:5[1]	<1:5	-
1944	<1:5	<1:5	-
1945	<1:5	<1:5	-
1946	<1:5	<1:5	-
1947	1:5[1]	<1:5	-
1948	<1:5	<1:5	-

1) A second examination gave a titer of < 1:5.

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP- Präzipitations-Test
1949	<1:5	<1:5	-
1950	<1:5	<1:5	-
1938	<1:5	1:30	-
1939	<1:5	1:7,5	+
		Bestand Win.	
892	<1:5	1:40	-
893	<1:5	n.u.	+
894	1:5	1:30	-
895	1:16	1:30	+
896	<1:5	n.u.	+
2169	1:8	1:20	+
2170	<1:5	<1:5	+
2171	<1:5	1:160	+
2172	<1:5	1:240	+
2173	<1:5	1:30	-
		Bestand Scho.	
972	1:5	n.u.	-
973	<1:5	1:20	-
974	<1:5	1:320	-
975	<1:5	1:320	-
976	<1:5	1:20	-
977	<1:5	1:640	+
2069	<1:5	1:30	-
2070	<1:5	1:30	-
2071	<1:5	1:40	-
2072	<1:5	1:20	-
2073	<1:5	1:20	-
2074	<1:5	1:60	-
2075	<1:5	1:15	-
2076	<1:5	1:320	-
2077	<1:5	1:20	-
2078	<1:5	<1:5	-
2079	<1:5	1:320	-

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP-Präzipitations-Test 1)
2080	<1:5	<1:5	-
2081	<1:5	<1:5	-
2082	<1:5	<1:5	-
2083	<1:5	<1:5	-

Key:
1) EHC-precipitation test; 2) Herd.

II. NIF-negative swine herds

A. Herds with cattle housing

Result of Examination

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP-Präzipitations-Test 1)
		Bestand 2) Gü.	
1482	<1:5	<1:5	+
1483	<1:5	<1:5	+
1484	<1:5	<1:5	-
1485	<1:5	1:7,5	+
1486	<1:5	>1:80	-
1487	<1:5	1:5	-
		Bestand 2) K.Br.	
1508	<1:5	<1:5	-
1509	<1:5	1:80	-
1510	<1:5	1:320	-
1511	<1:5	1:80	-
1512	<1:5	1:20	-
		Bestand 2) G.Br.	
1503	<1:5	<1:5	-
1504	<1:5	<1:5	+
1505	<1:5	<1:5	+
1506	<1:5	<1:5	+
1507	<1:5	<1:5	-
		Bestand 2) Ba.	
1488	<1:5	<1:5	-
1489	<1:5	1:10	-
1490	<1:5	1:10	-

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP-Präzipitations-Test
1491	< 1:5	1:15	-
1492	< 1:5	1:10	-
1493	< 1:5	1:10	-
1494	< 1:5	1:10	+
1495	< 1:5	< 1:5	+
1496	< 1:5	< 1:5	-
		Bestand Bi.	
1461	< 1:5	< 1:5	-
1462	< 1:5	1:10	+
1463	< 1:5	1:160	-
1464	< 1:5	1:10	-
1465	< 1:5	1:320	-
		Bestand Ger.	
1446	< 1:5	1:10	-
1447	< 1:5	< 1:5	-
1448	< 1:5	< 1:5	-
1449	< 1:5	1:10	-
1450	< 1:5	< 1:5	-
		Bestand Ga.	
1437	< 1:5	< 1:5	-
1438	< 1:5	< 1:5	-
1439	< 1:5	< 1:5	+
1440	< 1:5	1:10	-
1441	< 1:5	< 1:5	-
		Bestand O.	
1425	< 1:5	< 1:5	-
1426	< 1:5	< 1:5	+
1427	< 1:5	< 1:5	+
1428	< 1:5	1:40	-
1429	< 1:5	1:320	-
1430	< 1:5	< 1:5	-

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP-Präzipitations-Test 1)
	Bestand Ra. 2)		
1392	<1:5	< 1:5	-
1393	<1:5	< 1:5	-
1394	<1:5	< 1:5	-
1395	<1:5	<1:5	+
1396	<1:5	<1:10	+
	Bestand Hi. 2)		
1326	<1:5	< 1:5	+
1327	<1:5	< 1:5	+
1328	<1:5	<1:5	+
1329	<1:5	1:15	+
1330	<1:5	< 1:5	+

Key:
1) EHC-precipitation test; 2) Herd.

B. Herds without cattle housing

Result of Examination

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP-Präzipitations-Test 1)
	Bestand H.Wü. 2)		
705	<1:5	1:15	+
706	<1:5	1:7,5	-
707	<1:5	<1:5	-
708	<1:5	<1:5	-
709	<1:5	1:7,5	-
	Bestand L. 2)		
691	<1:5	<1:5	-
692	<1:5	1:10	-
693	<1:5	1:10	-
694	<1:5	<1:5	-
	Bestand Br. 2)		
878	<1:5	< 1:5	+
879	<1:5	< 1:5	+
880	<1:5	<1:5	+
881	<1:5	<1:5	+

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP-Präzipitations-Test
		Bestand ES.	
873	<1:5	<1:5	+
874	<1:5	<1:5	+
875	<1:5	1:15	+
876	<1:5	1:10	+
877	<1:5	1:10	+
		Bestand Eg.	
848	<1:5	1:40	-
849	<1:5	1:30	-
850	<1:5	1:60	-
851	<1:5	1:30	-
		Bestand Wü.	
843	<1:5	<1:5	-
844	<1:5	<1:5	-
845	<1:5	1:10	+
846	<1:5	1:10	-
847	<1:5	1:10	+
		Bestand M.	
838	<1:5	1:10	+
839	<1:5	<1:5	+
840	<1:5	1:10	-
841	<1:5	1:10	-
842	<1:5	<1:5	+
		Bestand Ke.	
687	<1:5	<1:5	-
688	<1:5	1:7,5	-
689	<1:5	<1:5	-
690	<1:5	1:160	-
		Bestand F.T.	
1761	<1:5	<1:5	-
1762	<1:5	1:30	+
1763	<1:5	1:30	-
1764	<1:5	<1:5	+
1765	<1:5	<1:5	-

Serum-No.	NIF-Test	BVD-MD-N-Test	ESP- Präzipitations-Test
		Bestand L.T.	
1312	<1:5	<1:5	+
1313	<1:5	<1:5	+
1314	<1:5	<1:5	-
1315	<1:5	<1:5	+
1316	<1:5	<1:5	-
1317	<1:5	<1:5	+
1318	<1:5	<1:5	-
1319	<1:5	<1:5	+
1320	<1:5	<1:5	-
1321	<1:5	1:640	+
1322	<1:5	1:20	+
1323	<1:5	<1:5	-
1324	<1:5	1:20	+
1325	<1:5	<1:5	+

Key:
1) EMC-precipitation test; 2) Herd.

Culture media and salt solutions

<u>Salzlösung nach Hanks</u> 1

NaCl	8,00 g
KCl	0,40 g
$CaCl_2$	0,14 g
$Mg\ SO_4 \times 7\ H_2O$	0,20 g
$Na_2HPO_4 \times 12\ H_2O$	0,12 g
KH_2PO_4	0,06 g
$NaHCO_3$	0,35 g
Glucose	1,00 g
Aqua bidest	ad 1000 ml
pH 7,2 - 7,4	

<u>Anzuchtmedium</u> 2

1 Salzlösung nach Hanks	90 ml
3 Kälberserum	10 ml
Penicillin	100 I.E. pro ml
Streptomycin	0,1 mg pro ml

4 <u>Salzlösung nach Earle</u>

NaCl	6,80 g
KCl	0,40 g
$CaCl_2$	0,20 g
$Mg\ SO_4 \times 7\ H_2O$	0,20 g
$Na_2HPO_4 \times 12\ H_2O$	0,125 g
$NaHCO_3$	2,20 g
Glucose	1,00 g
Aqua bidest	ad 1000 ml
pH 7,4	

5 <u>Erhaltungsmedium</u>

4 Salzlösung nach Earle	95 ml
3 Kälberserum	5 ml
Penicillin	100 I.E. pro ml
Streptomycin	0,1 mg pro ml

6 <u>Phosphat-Pufferlösung</u> (ohne $CaCl_2$ und $MgCl_2$)

NaCl	8,00 g
KCl	0,20 g
$Na_2HPO_4 \times 12\ H_2O$	2,37 g
KH_2PO_4	0,20 g
Aqua bidest	ad 1000 ml
pH 7,4	

PBS-Versenlösung (7)

NaCl	8,00 g
KCl	0,20 g
KH_2PO_4	0,20 g
Na_2HPO_4 x 12 H_2O	2,90 g
EDTA	0,20 g
Aqua bidest	ad 1000 ml
pH 7,4	

PBS-Versen-Trypsinlösung (8) (0,05 % Trypsin-Lösung)

PBS-Versen-Lösung (7)	95 ml
1%ige Trypsin-Lösung in Ca-Mg-freier PBS (9)	5 ml

Natrium-Barbital-Puffer (10)

Natriumdiäthylbarbiturat (11)	2,58 g
Diäthylbarbitursäure (12)	0,46 g
Natriumazetat (13)	1,70 g
Aqua bidest	ad 1000 ml
pH 8,6; Ionenstärke 0,025	

Agar-Lösung (14)

Spezial Agar-Noble (15) (Fa. Difco,Detroit/USA)	1,00 g
Natrium-Barbital-Puffer (16)	ad 10C ml

Haftagar-Lösung (17)

1% Agarlösung (18)	10 ml
Glycerin	1,5 ml
Aqua bidest	ad 100 ml

Extraktionsmedium (19)

Harnstoff (20)	60,06g
EDTA	0,148 g
Tris	12,1 g
Aqua bidest	ad 1000 ml

Key:
1) Salt solution after Hanks; 2) Growth medium; 3) Calf serum; 4) Salt solution after Earle; 5) Maintenance medium; 6) Phosphate buffer solution (without $CaCl_2$ and $MgCl_2$; 7) PBS-Versene-solution; 8) PBS-Versene trypsin solution (0.05% trypsin solution); 9) 1% trypsin solution in Ca-Mg-free PBS; 10) Sodium-barbital-buffer; 11) Sodium diethyl barbiturate; 12) Diethyl barbitaric acid; 13) Sodium acetate; 14) Agar solution; 15) Special agar "Noble"; 16) Sodium carbital buffer; 17) Adhesive agar solution; 18) 1% agar solution; 19) Extraction medium; 20) Urea.

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